

REMARKS

Claims 1-5, 10-14, 21, 23-26, 28, and 29 are being examined in this application. These claims stand rejected under 35 U.S.C. § 112, first paragraph, and 35 U.S.C. § 103. Each of these issues is addressed below in the order in which it appears in the Office action.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 1-5, 10-14, 21, 23-26, 28, and 29 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. These rejections are addressed as follows.

The Office's first basis for its enablement rejection is the Office's contention that the use of an "immunoglobulin gene" is not enabled because there is not a gene for each antibody found in an animal's repertoire. Applicants respectfully disagree with the position taken by the Office. "Immunoglobulin gene" refers to a gene encoding an immunoglobulin protein chain (e.g., a heavy chain or a light chain). That the germline genes undergo a recombination process that joins a number of different gene segments does not negate the existence of the immunoglobulin gene. However, as proposed by the Office, applicants have amended claims 1 and 10 (which referred to an "immunoglobulin gene") and 21 and 26 (which referred to a "gene locus"), as well as claims dependent therefrom, to instead refer to "an immunoglobulin locus." It is applicants' position that this term similarly refers to a gene encoding an immunoglobulin protein chain, and in fact

applicants have used the term immunoglobulin locus in this manner in the present specification (see, for example, page 40, lines 24-25: “the Mu locus (corresponds to IgM heavy chain gene)”). This basis for the rejection may be withdrawn.

The Office also contends that the claims lack enablement because they encompass methods employing part of an immunoglobulin gene, while the specification provides no guidance as to which parts of the gene are needed for antibody production. Applicants have amended the claims; they no longer read on use of a part of an immunoglobulin gene. Applicants reserve the right to pursue this subject matter in this or a related application.

As a third basis for rejecting the claims for lack of enablement, the Office states that “the vector used to introduce the Ig locus is critical to the production of the claimed ungulates and ultimately to the production of xenogeneic antibodies” and asserts that the method of making the immunoglobulin-producing animals must be recited in the claims. The Office acknowledges that applicants’ specification enables methods that make use of artificial chromosomes or micro cell type vectors. Applicants have amended the claims to recite that the immunoglobulin locus is contained within an artificial chromosome, and this rejection may now be withdrawn.

As a yet further basis for the enablement rejection, the Office notes that the specification does not describe the rearrangement of an immunoglobulin locus such that antibody would be produced, and questions whether (i) the xenogenous antibody locus

would undergo rearrangement; (ii) antibody would be produced; and (iii) the artificial chromosome would be retained. On this issue, applicants direct the Office's attention to the attached paper by Kuroiwa et al. (Exhibit A; Nature Biotechnol. 20:881-882, 2002), discussing applicants' work and describing the production of transchromosomal calves producing human immunoglobulin. The calves described by Kuroiwa were made using the methods described in the specification. The Kuroiwa authors found that (i) the Ig loci underwent rearrangement and expressed diversified transcripts, (ii) human immunoglobulin proteins were produced and were detected in the blood of newborn calves, and (iii) the immunoglobulin locus-containing artificial chromosome was retained at a high rate (78-100% of calf cells; see Abstract). Thus, as demonstrated by this publication describing applicants' work, each of the concerns raised by the Office relating to antibody production is unwarranted, and this basis for the enablement rejection should also be withdrawn.

Finally, the Office contends that "any antibody that contains a non ungulate Ig would necessarily be a hybrid ungulate-nonungulate antibody" that would have no therapeutic value because "the bovine portion of a human-bovine antibody will itself induce an immune response when administered to a human." On this basis, the Office concludes that the claimed animals and methods have no use. Applicants respectfully traverse this basis for the rejection.

Applicants first note that the Office's conclusion that the produced antibody will

inherently be a hybrid antibody is incorrect. Indeed, applicants have found that transgenic cattle engineered to produce human IgG express three different types of IgG molecules: bovine IgG (uIgG), human IgG (hIgG), and chimeric or hybrid IgG (cIgG) that contains either human heavy chain (HC) and bovine light chain (LC) or human LC and bovine HC. Applicants have determined that the concentration of hIgG in transgenic bovine plasma ranges from 10 to 30 µg/ml, while bIgG concentration is in the range of 10-20 mg/ml, and the concentration of cIgG is unknown. Serum may be collected from these ungulates and stored for later human antibody production. Moreover, if desired, the level of human immunoglobulin in the serum may be increased by disrupting expression of ungulate immunoglobulin genes, as described in applicants' specification at pages 40-47. Thus, contrary to the Office's assertion, applicants' claimed ungulates do produce human antibody, and do have a use. This basis for the enablement rejection should also be withdrawn.

For all of the foregoing reasons, applicants submit that the claims as amended are enabled. The § 112, first paragraph rejections may be withdrawn.

Rejection under 35 U.S.C. § 103(a)

The claims stand further rejected under 35 U.S.C. § 103 as being unpatentable over Lonberg (U.S. Patent No. 5,569,825) in view of Deboer (U.S. Patent No. 5,741,957). The Office asserts that it would have been obvious to produce transgenic bovines by

combining Lonberg's mini-gene locus with Deboer's methods of producing transgenic bovines. Without assenting to any of the statements made by the Office, applicants submit that the claims as amended are not obvious over the combination of Lonberg and Deboer.

The claims as amended each require an ungulate having at least one artificial chromosome that contains at least one immunoglobulin locus. An artificial chromosome is an autonomous, replicating DNA containing a centromere, two telomeres, and an origin of replication.

In order to render the claims obvious, some combination of the cited prior art must teach or suggest every limitation of the claimed invention. M.P.E.P. 2142. In the present case, neither Lonberg nor Deboer teach or suggest the use of an artificial chromosome. In each case, the DNA being introduced into the host animal in the cited references is a transgene designed to integrate into the host genome, and thus it does not remain autonomous. Additionally, the transgene lacks a centromere and telomere, as well as an origin of replication.

Because the combination of Lonberg and Deboer fails to teach or suggest the claimed invention, applicants submit that the obviousness rejection may be withdrawn.


CONCLUSION

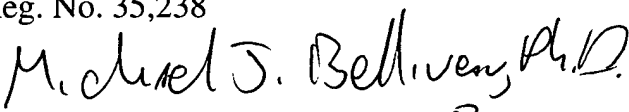
Applicants submit that the claims are in condition for allowance, and such action is respectfully requested.

Enclosed is a Petition to extend the period for replying to the Office action for three months, to and including January 29, 2005, and a check in payment of \$1020.00 for the required extension fee. If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 1/28/05


Karen L. Elbing, Ph.D.
Reg. No. 35,238


Reg. No. 52,608

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Exhibit A

RESEARCH ARTICLE

Cloned transchromosomal calves producing human immunoglobulin

Yoshimi Kuroiwa¹, Poothappillai Kasinathan², Yoon J. Choi³, Rizwan Naeem⁴, Kazuma Tomizuka¹, Eddie J. Sullivan², Jason G. Knott², Anae Duteau³, Richard A. Goldsby³, Barbara A. Osborne⁵, Isao Ishida^{1*} and James M. Robl^{2*}

Published online: 12 August 2002, doi:10.1038/nbt727

Human polyclonal antibodies (hPABs) are useful therapeutics, but because they are available only from human donors, their supply and application is limited. To address this need, we prepared a human artificial chromosome (HAC) vector containing the entire unrearranged sequences of the human immunoglobulin (hlg) heavy-chain (H) and lambda (λ) light-chain loci. The HAC vector was introduced into bovine primary fetal fibroblasts using a microcell-mediated chromosome transfer (MMCT) approach. Primary selection was carried out, and the cells were used to produce cloned bovine fetuses. Secondary selection was done on the regenerated fetal cell lines, which were then used to produce four healthy transchromosomal (Tc) calves. The HAC was retained at a high rate (78–100% of cells) in calves and the hlg loci underwent rearrangement and expressed diversified transcripts. Human immunoglobulin proteins were detected in the blood of newborn calves. The production of Tc calves is an important step in the development of a system for producing therapeutic hPABs.

Despite the substantial need for hPABs to treat many diseases, the supply is limited to what can be obtained from human donors. Furthermore, the application of hPABs has been restricted because human donors cannot be hyperimmunized, that is, repeatedly boosted with antigen. Transgenic animals carrying hlg loci could provide a source of hPABs, especially targeted hPABs resulting from hyperimmunization with human pathogens or human molecules. Transgenic mice carrying hlg loci have been created^{1–6} and are useful for the derivation of human monoclonal antibody therapeutics^{7,8}.

Transgenic cattle carrying hlg loci could be useful for large-volume commercial production of hPABs. Transgenesis including gene targeting^{9–12} in livestock has been reported; however, the procedures used are not suitable for transfer of the hlg loci¹³ (1–1.5 Mb for each locus) because the maximum size of DNA that can be inserted is very limited (20–100 kb). Mammalian artificial chromosome (MAC) vectors^{14–17} may be a better choice because of their large insert capacity. Thus far, there have been no reports of the transfer of MAC vectors in livestock. Furthermore, human microchromosomes are generally mitotically unstable in a foreign environment^{11,18,19}. This could be a major obstacle in the production of transchromosomal cattle, which require a large number of cell divisions for full term development.

Another potential limitation of using cattle to produce hPABs is the difference in immunophysiology between cattle and humans^{19–22}. In humans and mice, bone marrow is the major site of origin of all lymphocytes and the location of subsequent B-cell maturation. In contrast, spleen, rather than bone marrow, is the presumed site of B-cell origin and immunoglobulin rearrangement in bovine. Furthermore, because of a limited number of

functional V genes in bovine, gene conversion may be an important mechanism for the generation of diversity, especially for the light chain^{19,20}. Gene conversion occurs in the ileal Peyer's patch, where B cells undergo proliferation and diversification. These differences could impede the functional rearrangement, diversification, and production of hlg in cattle.

In this study, we developed a system for introducing heavy- and light-chain hlg loci into bovine by transferring a 10 Mb HAC vector carrying the loci into primary fibroblast cells and then producing cloned cattle from the Tc cells. We also evaluated the retention of the HAC through early gestation and the functional rearrangement, diversification, and expression of hlg in the blood of Tc calves.

Results

HAC transfer into bovine fetal fibroblasts and nuclear transfer. We constructed two HAC vectors (ΔHAC and ΔΔHAC), each carrying both hlg heavy-chain and λ light-chain loci, using a chromosome-cloning system^{17,23} (Fig. 1). HAC vectors were introduced into bovine primary fetal fibroblasts from CHO clones using an MMCT system (Fig. 2). The life-span limits of bovine primary fibroblast cells required that complete antibiotic selection and DNA-based screening be avoided after MMCT to minimize cell divisions before nuclear transfer. Instead, we picked colonies on the basis of growth and morphology under selection and used them for nuclear transfer as quickly as possible. Nevertheless, the cells were useful only for a few days and could not be cryopreserved. Final selection was done after the rejuvenation and expansion of the cells during the growth of cloned fetuses. At 36–58 days, four

¹Pharmaceutical Research Laboratory, Kirin Brewery Co., Ltd., 3 Miyahara-cho Takasaki-shi Gunma 370-1295, Japan. ²Hematech, LLC, 33 Riverside Avenue, Westport, CT 06880. ³Department of Biology, Amherst College, Amherst, MA 01002. ⁴Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. ⁵Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA 01003. *Corresponding authors (jrobl@hematech.com and i-ishida@kirin.co.jp).

RESEARCH ARTICLE

Table 1. Development of cloned embryos derived from Tc fibroblasts

HAC	Nuclear transfer	Blastocysts (%) ^a	Blastocyst transfer	Recipients	Pregnant 40 days (%)	Pregnant 120 days (%)	Offspring (%) ^b
Δ	770	83 (10)	34	32	16 (50)	Fetal recovery	0
ΔΔ	833	122 (21)	58	28	13 (48)	Fetal recovery	1
Δ Regenerate	672	82 (17)	61	37	8 (22)	8 (18)	6 (16)

Fetal recovery was done between 56 and 119 days of gestation. Regenerated cells were produced from Tc fetuses recovered at 56–58 days of gestation.

^aPercentage of blastocysts was calculated as the number of blastocysts per number of fused donor cell oocyte complexes. Average fusion rate in our laboratory was 70%.

^bFor ΔΔHAC nuclear transfers, one fetus was not recovered and developed to term producing a live healthy calf. For ΔHAC regenerated cells, six live calves were born; two from cell line 6032 did not survive past 48 h and the other four remain alive and healthy.

ΔHAC and two ΔΔHAC fetuses were recovered and fibroblast cell lines were regenerated, expanded, and cryopreserved for further analysis and nuclear transfer. Efficiency of development to the blastocyst stage, to 40 days of gestation, and to term is shown in Table 1 for first-generation and re-cloned cells.

Analysis of cloned Tc fetuses. To examine whether the HAC vector was retained through early gestation and whether *hlg* loci could be functionally rearranged and expressed during early B-cell development in bovine, we analyzed cloned Tc fetuses collected between 56 and 119 days of gestation. Retention of the HACs in the fibroblast lines derived from six cloned Tc fetuses collected at 56–58 days and from an additional seven fetuses collected between 77 and 119 days was evaluated by G418 resistance (Fig. 3A) and by genomic PCR of human *IgH* and *Igλ* loci (Fig. 3B). Of the 13 fetuses, 9 were resistant to G418 (4 ΔHAC and 5 ΔΔHAC) and 8 showed the presence of both human *IgH* and *Igλ* loci. We evaluated five positive 77–119-day fetuses for expression and rearrangement of the *hlg* loci by reverse transcription-PCR (RT-PCR) analysis, followed by sequencing of the amplified products. Human *IgH* and *Igλ* genes were expressed (Fig. 3C) in all fetuses, predominantly in spleen, consistent with endogenous bovine *Ig*

expression (data not shown). The sequences showed evidence of functional V(D)J recombination (Fig. 3D). These results demonstrate retention of the HAC vector, functional V(D)J recombination, and expression of the *hlg* locus.

Generation of cloned Tc calves. For production of cloned Tc calves, the three regenerated ΔHAC cell lines (5968, 6032, and 6045) were used for recloning. One male calf from cell line 6045 and five female calves from cell lines 5968 and 6032 were produced from 37 recipients (16%, Fig. 4A). Four calves survived and were healthy and phenotypically normal. Retention of the HAC vector was confirmed in all the calves by G418 selection (data not shown), genomic PCR (Fig. 4B), and fluorescent *in situ* hybridization (FISH) analyses (Table 2; Fig. 4C). FISH analysis indicated that the HAC was retained as an independent chromosome and that the proportion of cells retaining the HAC ranged from 78% to 100%. We observed no obvious differences in retention rates between peripheral blood lymphocytes (PBLs: 91%) and fibroblasts (87%). These data demonstrate that somatic-cell recloning strategies can be used to produce healthy cloned Tc calves and that the HAC vector can be stably maintained through the large number of cell divisions in bovine development.

Human *Ig* gene expression and protein production in Tc calves. To determine whether *hlg* loci were rearranged and expressed, we carried out RT-PCR analysis on PBLs. We observed expression of both human *IgH* and *Igλ* genes in the PBLs of all the calves (data not shown). The diversity of the human *IgH* and *Igλ* repertoire was determined by sequence analysis (Table 3). A representative set of the sequences showed a wide utilization of VH/VL, D, and JH/JL segments distributed over the loci. In the *IgH* transcripts, the frequent utilization of JH4 and of V segments from VH1 and VH3 was observed, similar to patterns in human¹⁴. Addition of non-germline nucleotides (N addition) and nucleotide deletion were also observed in both *hlgH* and *hlgλ* transcripts. These produced a high degree of diversification in the third complementarity-determining region of both the *hlgH* and *hlgλ* chains. Furthermore, *hlg* proteins were secreted at levels ranging from 13 ng/ml to 258 ng/ml (immunoglobulin expression is typically very low to undetectable in newborn calves²⁵) in blood samples collected before colostrum feeding in five of the seven Tc calves. In the two calves in which *hlg* proteins were not detected, bovine

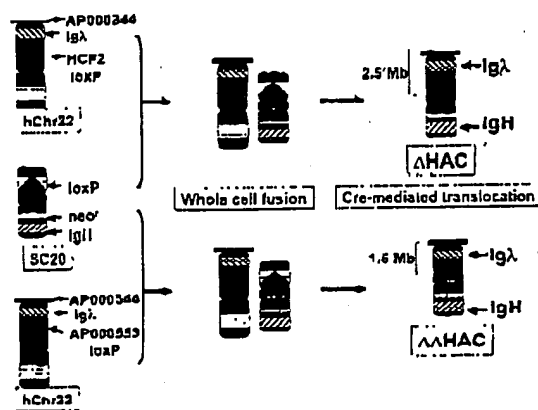


Figure 1. Diagram of ΔHAC and ΔΔHAC construction. In ΔHAC construction, the hChr22 fragment (hChr22), which contains the *Igλ* locus, was truncated at the AP000344 locus and a *loxP* sequence was integrated at the HCF2 locus. In ΔΔHAC construction, the *loxP* sequence on the hChr22 fragment was integrated at the AP000653 locus. DT40 cells containing the hChr22 fragments were fused with DT40 cells containing the SC20 fragment (SC20 is a fragment of hChr14 and contains the *IgH* locus). Cre-mediated translocation resulted in ΔHAC, which contained a 2.5 Mb hChr22 region, and ΔΔHAC, which contained a 1.6 Mb hChr22 region, each fused to the SC20 vector.

Table 2. HAC retention in cloned Tc calves

Calf number	Cell type	HAC positive/total (%)	Two signals/total (%)
50	PBL	50/50 (100)	6/50 (12)
50	Fibroblast	47/50 (94)	0/50 (0)
1094	PBL	40/50 (82)	0/50 (0)
1064	Fibroblast	34/39 (87)	3/39 (8)
1055	PBL	39/50 (78)	2/50 (4)
1055	Fibroblast	49/50 (98)	0/50 (0)
1066	PBL	47/50 (94)	1/50 (2)
1066	Fibroblast	43/50 (86)	0/50 (0)
Total	Combined	355/399 (89)	12/399 (3)

Retention rate was determined in both PBLs and fibroblasts in each calf by FISH analysis using human Cot-I DNA as a probe.

RESEARCH ARTICLE

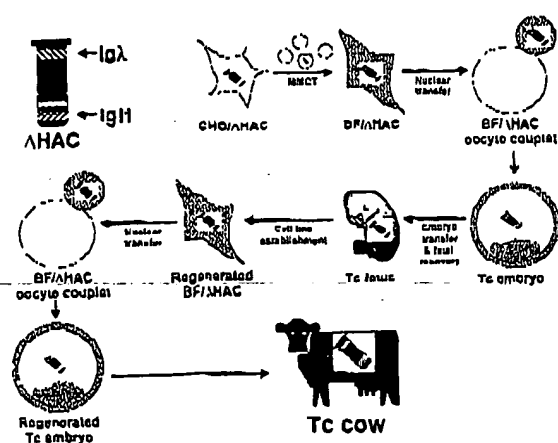


Figure 2. Procedure for production of cloned Tc calves. Structure of Δ HAC (upper left) is shown with hChr22 (green) and hChr14 (red) regions containing *Ig* λ and *Ig*H loci. The centromere of the HAC is derived from the hChr14 fragment. The HAC was transferred from a CHO clone (CHO/ Δ HAC, light blue) into fetal bovine fibroblasts by means of a MMCT technique. Tc fibroblasts (BF/ Δ HAC, black patterned) and enucleated oocyte (yellow) couplets were fused, resulting in transfer of the fibroblast nucleus and formation of an embryo. The reconstituted Tc embryos were cultured *in vitro* to the blastocyst stage and then implanted into recipient cows. At ~80 days of gestation, Tc fetuses were recovered and Tc fibroblast cell lines were re-established (regenerated BF/ Δ HAC), evaluated, and used for further nuclear transfer. Regenerated Tc embryos were transferred to recipients to produce Tc calves.

immunoglobulin proteins were also not detected. These results demonstrate that the human *IgH* and *Ig* λ loci can be functionally rearranged and expressed in cattle.

Discussion

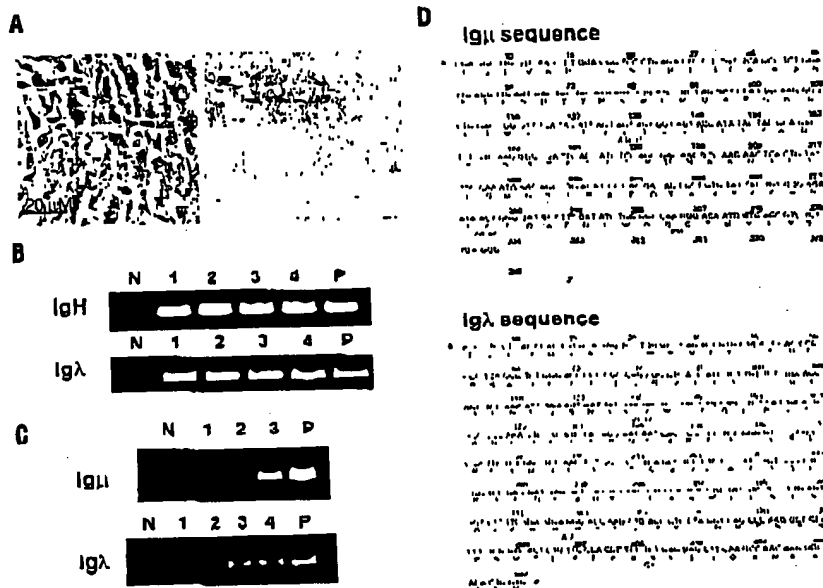
In this study, we demonstrate the production of healthy calves carrying a stable microchromosomal vector encoding human *IgH* and *Ig* λ loci, functional diversification of the *hlg* genes, and production of *hlg* protein. To accomplish this, several challenges needed to be addressed, including the lack of methods for introducing large foreign DNA sequences into cattle, the possible mitotic instability of a microchromosome vector, and the substantial differences in immune system development between cattle and humans.

MMCT has been accomplished in mice using embryonic stem cells and chimera production followed by germline transmission from embryonic stem cell-derived germ cells¹. Mouse embryonic

stem cells have the advantage of an unlimited life span, rapid growth in culture, a straightforward system for clonal propagation of transgenic colonics, and a high rate of contribution to the germ line in chimeras. Unfortunately, embryonic stem cells with similar properties have not been derived in other species, including cattle²⁴. It was therefore necessary to use a nuclear transfer system with primary somatic cells²⁷ to produce Tc cattle. The limitation of this system is that primary fibroblasts, the cell type of choice, have a life span of ~35 population doublings²⁸. After MMCT, our cells were capable of dividing for only about one week and could not be cryopreserved. Our approach to solving this problem was to carry out incomplete selection after MMCT and complete selection on rejuvenated cloned cell lines, and then produce calves by recloning cell lines. Recloning of transgenic somatic cells and production of offspring has been accomplished in cattle²⁹ with limited success. Our results indicate that rejuvenation of cell lines by recloning is a viable method for the production of large numbers of transgenic animals and the establishment of cryopreserved transgenic cell banks.

In mice, the SC20 HAC vector is retained in 70–80% of fibroblasts¹ and only 30–40% of PBLs (data not shown), revealing limitations in mitotic stability in a foreign environment. Because of the number of cell divisions required for bovine development, the retention rate of the HAC was expected to be low. However, the HAC was retained in all cloned calves at a very high rate, both in

Figure 3. Analysis of Tc fetuses. (A) G418 selection of regenerated Tc fibroblast line (left) and control nontransgenic fibroblasts (right). (B) Genomic PCR of *IgH* and *Ig* λ loci in Tc fetuses and controls. The three fetuses, 5568 (lane 1), 5532 (lane 2), and 5045 (lane 3) were derived from Δ HAC fibroblasts; fetus 5580 (lane 4) was derived from Δ HAC fibroblasts. As a control, a nontransgenic fetus (lane N) was recovered and evaluated. Human *IgH* and *Ig* λ loci were detected by PCR in all Tc fetuses and in a positive-control human liver DNA sample (lane P), but not in the negative control (lane N). (C) Rearranged and expressed human *Ig* μ and *Ig* λ transcripts amplified by RT-PCR from negative-control nontransgenic bovine spleen (lane N), from brain (lane 1), liver (lane 2), and spleen (lane 3 and 4) of cloned Tc fetus, and from positive-control human spleen (lane P). (D) A representative nucleotide and deduced amino acid sequence of human *Ig* μ and *Ig* λ transcripts amplified by RT-PCR from a cloned Tc fetus recovered at 89 days. In the *Ig* μ transcript, blue represents the variable region sequence (VH3-11), red represents the diversity region (D7-27), green represents the joining region (JH3) and orange represents the constant region. In the *Ig* λ transcript, blue represents the variable region sequence (VL1-17), red represents the joining region (JL3), and green represents the constant region sequence.



RESEARCH ARTICLE

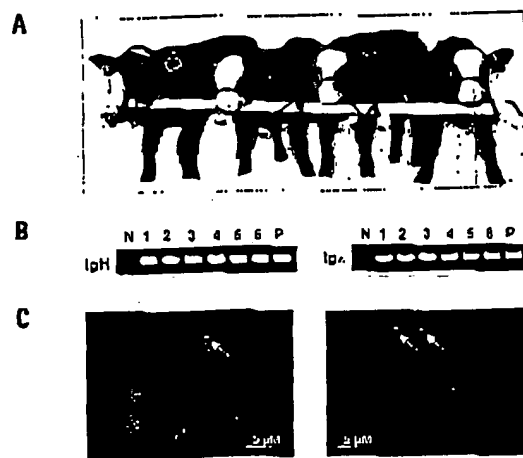


Figure 4. Analysis of cloned Tc calves. (A) Four cloned Tc calves: male calf (50) from cell line 6045 and female calves (1064, 1065, 1066) from cell line 5968. (B) Genomic PCR of *IgH* and *IgL* loci in PBLs from cloned Tc calves and controls: calf 1064 (lane 1), 1065 (lane 2), 1066 (lane 3), 60 (lane 4), 1067 (lane 5), and 1068 (lane 6). Both human *IgH* and *IgL* loci were detected by genomic PCR in all the Tc calves and positive-control human liver DNA (lane P), but not in a negative-control nontransgenic calf (lane N). (C) FISH analysis in metaphase chromosome spreads in a cell showing a single signal and a cell showing a double signal. Arrows indicate location of HACs (red) among surrounding bovine chromosomes (blue). A single HAC per cell is introduced and retained in most cells (left panel); however, improper separation of chromatids at cell division may result in some cells having two microchromosomes (right panel) and some not having a microchromosome.

fibroblasts (87%) and PBLs (91%). In our previous study¹⁷, the mitotically unstable hChr22 fragment was mitotically stabilized when its centromere was exchanged with that of the SC20 vector, suggesting that the mitotic stability of human microchromosomes might be affected by centromeric structure. In this study, the unexpectedly high mitotic stability of the HAC vector in bovine may be explained by greater compatibility of the human cen-

tromeric structure with factors regulating cell division in cattle as compared with mice. The high rate of HAC retention shows the utility of HAC vectors for stable introduction of foreign genetic elements into the genome of cattle.

We have shown that *hlg* loci can be functionally rearranged and expressed with substantial diversity in cattle despite the differences in immunophysiology between cattle and humans. Both broad usage of VDJ segments and N addition contributed considerably to *hlg* diversity in human *IgH* and *IgL* transcripts in cloned Tc calves. Notably, in bovine *IgL* transcripts, N addition is rarely observed (data not shown). These results indicate that immunoglobulin gene diversification may be more related to genetic sequence than to the environment in which diversification occurs.

Table 3. Repertoire analysis of human immunoglobulin heavy- and λ -chain transcripts in cloned Tc calves

Human μ nucleotide sequences				
VH	N	DH	N	JH
8-1	0	D5-24	3	JH3
TACTGTGCA		AGAGATG	AGA	ATGCTTTTGATGTC
3-33	8	D6-13	3	JH4
ATTACTGTGCGA	AGAACAAA	ATAGCAGCAGCTGGTAC	GAT	CTTTGACTACT
3-16	4	D8-19	4	JH1
ACTGTACCAACAGA	TCTG	ATAGCAGTGGCTGGTAC	TGGG	TACTTCCAGCA
3-66	2	D2-2	0	JH2
TACTGTGCGAG	TC	GTAGTACCAGCTGCTAT		GATGCTTTTGATGTCT
3-21	8	D2-21	8	JH6
TTACTGTGCCAG	TTTTGG	GTGGTGGT	CACATTTA	GACTACTGGGG
4-39	8	D3-10	3	JH4
ACTGTGCCAGACA	TGAAAAAC	TTCGGGGAGTTAT	AAT	CTACTGGGGCC
1-69	7	D8-13	1	JH4
TTACTGTGCGAG	GGGGATG	GCAGCAGCTGGTAC	C	GACTACTGGGGC
1-8	0	D2-2	12	JH2
ACTGTGCCAGAC		ATTGTAGTAGTACCAGCTGC	CAAGATCGTAAG	TGGTACTTCGAT
1-18	0	D5-24	15	JH4
TTACTGTGC		GAGATGG	GTTTTTGATCCCCAG	TTTGACTACTGG
3-20	4	D7-27	1	JH3
TCACTGTGCCAGAA	TTTT	ACTGGGGA	T	GATGCTTTTGATGTCT
Human λ nucleotide sequences				
VL	N	JL		
1-17	2 (TT)	JL3	TTGGCGGAGGG	
2-13	0	JL2	GGTATTCGGCGGAGG	
1-19	0	JL1	TCTTCGGAACTGGG	
5-2	2 (TA)	JL3	GTTTCGGCGGAGAG	
1-7	1 (G)	JL3	TGGCGGAGGGA	
2-13	0	JL1	TATGCTTCGGAAGT	
2-1	0	JL1	TATGCTTCGGAAGT	
1-2	1 (G)	JL1	ATGCTTCGGAAGT	
1-4	2 (GT)	JL3	TTGGCGGAGG	
1-4	0	JL1	GGAACTGGGA	

Human μ - and λ -specific mRNAs were amplified by RT-PCR, cloned, and sequenced. Nucleotide sequences of VDJ junctions of each of ten independent μ and λ clones are shown, divided into V μ /V λ , D, J μ /J λ , and N segments, as identified by homology to published germline sequences (Ig-BLAST).

RESEARCH ARTICLE

Several challenges remain before our system can be used for large-scale production of hPABs. Because Tc cattle retain the bovine Ig loci, expression of bovine antibody is expected to dominate over that of human antibody. We have observed this previously in Tc mice in which the murine Ig loci were not inactivated (data not shown). Furthermore, chimeric antibodies containing combinations of human and bovine heavy- and light-chain proteins are expected to be present. Therefore, methods of reducing bovine Ig expression are probably needed before commercial production of hPABs can proceed in Tc cattle.

A Tc bovine-based system for producing therapeutic hPABs would have several advantages: (i) cattle could be hyperimmunized with essentially any human pathogen or human molecule; (ii) cattle produce very large quantities of antibodies; (iii) large numbers of antigens could be evaluated quickly because one line of genetically modified cattle could be used for all antigens; and (iv) scale-up of antibody production would be as straightforward as immunizing additional cows. Therapeutic hPABs produced in a Tc bovine-based system may have broad application in the treatment and prevention of infectious disease (including antibiotic resistant infections), autoimmune disease, and cancer.

Experimental protocol

Construction of HAC vectors. HACs were constructed using a previously described chromosome-cloning system^{17,20} (Fig 1). Briefly, for the construction of Δ HAC, the previously reported hChr22 fragment (hChr22) containing a loxP sequence integrated at the HCF2 locus was truncated at the AP000344 locus by telomere-directed chromosomal truncation. Next, cell hybrids were formed by fusing the DT40 cell clone containing the hCF22 with a DT40 cell clone containing the stable and germline-transmissible human microchromosome vector SC20. The resulting DT40 cell hybrids contained both hChr fragments. The DT40 hybrids were transfected with a Cre recombinase expression vector to induce Cre-loxP-mediated chromosomal translocation between hChr22 and the SC20 vector. The stable transfectants were analyzed using nested PCR to confirm the cloning of the 2.5 Mb hChr22 region into the loxP-cloning site in the SC20 vector (Δ HAC). Δ HAC was constructed using the same chromosome cloning system except that the loxP sequence on hChr22 was integrated into the AP000333 locus, creating a 1.5 Mb insert upon Cre-loxP-mediated translocation.

HAC vector transfer into bovine fetal fibroblasts. Bovine fetal fibroblasts were cultured in α -MEM (Life Technologies, Rockville, MD) medium supplemented with 10% (vol/vol) FCS (Life Technologies) at 37°C and 5% CO₂. Microcells were purified from the CHO clone retaining the Δ HAC or Δ HAC as described previously¹⁹. Bovine fetal fibroblasts were fused with microcells using polyethylene glycol (PEG 1500, Roche, Nutley, NJ), and the fused cells were selected under 700 μ g/ml of G418 (Life Technologies) for 10–14 days. The G418-resistant clones were picked and used for nuclear transfer.

Nuclear transfer. The nuclear transfer procedure was carried out essentially as described previously^{17,20}. *In vitro*-matured oocytes were enucleated

–18–20 h post maturation. Cytoplasm-donor cell couplets were fused using a single electrical pulse of 2.4 kV/cm for 20 μ s (Electrocell Manipulator 200, Genetronics, San Diego, CA). At 30 h, post maturation reconstructed oocytes were activated with calcium ionophore (5 μ M) for 4 min (Cal Biochem, San Diego, CA) and 10 μ g cycloheximide and 2.5 μ g cytochalasin D (Sigma) as described earlier²¹. After activation, cloned embryos were placed in culture in four-well tissue culture plates, containing irradiated mouse fetal fibroblasts and 0.5 ml of ACM culture medium covered with mineral oil (Sigma) and incubated at 38.5°C in a 5% CO₂ in air atmosphere. On day 4, 10% (vol/vol) FCS (Life Technologies) was added to the culture medium. On days 7 and 8, embryos were transferred into synchronized recipients. All animal work was done following a protocol approved by the Trans Ova Genetics (Sioux Center, IA) Institutional animal care and use committee.

Genomic PCR analysis. Genomic DNA was extracted from Tc fetuses at 56–119 days of gestation or from cloned newborn calves and subjected to PCR using primers IGHV3 for the human IgH locus and IGLC for the human IgL locus, as described previously¹⁹.

RT-PCR and repertoire analyses. Total RNA was recovered from spleen, liver, and brain of Tc fetuses or from PBLs of Tc calves. RT-PCR was carried out as described previously¹⁹. For human IgM transcripts, VH1/5 BACK, VH3 BACK, and VH4BACK were used as a 5' primer and Cp-2 was used as a 3' primer. For human IgA transcripts, VA1LEA1, VA2MIX, and VA3MIX were used as a 5' primer and C4MIX was used as a 3' primer. The amplified cDNAs were subcloned by using a TA cloning kit (Invitrogen, San Diego, CA) and sequenced using a DNA autosequencer (ABI3700 Sequencer, ClaxoWellcome, Herts, United Kingdom).

FISH analysis. Digital image analysis was done using the Mac Probe system (Applied Imaging, Santa Clara, CA). HAC painting was done using digoxigenin-labeled (Boehringer Ingelheim, Ridgefield, CT) human Cor-1 DNA as a probe, and the digoxigenin signal was detected with an anti-digoxigenin-rhodamine complex that fluoresces red. DAPI (Sigma) was used for background staining. Standard chromosome and FISH protocols were carried out as described²¹.

ELISA analysis for human antibody. Plasma samples were obtained from Tc calves before they were fed colostrum and human Ig levels were determined by solid-phase ELISA. The assay used a bovine anti-human immunoglobulin as the capture antibody and an HRP-labeled sheep anti-human immunoglobulin as the detecting antibody. Amounts of human immunoglobulin >10 ng/ml were reliably detected by this assay.

Acknowledgments

We thank J. Pommer, J. Koster, J. Molina, and D. Faber at Trans Ova Genetics for their assistance with animals and T. King and M. Nichols for their assistance in nuclear transplantation. Partial support for work in the laboratory of R.A.C. was contributed by National Science Foundation grant NSF-MCB-9986213.

Competing interests statement

The authors declare competing financial interests; see the Nature Biotechnology website (<http://www.nature.com/naturebiotechnology>) for details.

Received 2 May 2002; accepted 4 June 2002

- Tomizuka, K. et al. Double trans-chromosome mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. *Proc. Natl. Acad. Sci. USA* **97**, 722–727 (2000).
- Bruggemann, M. et al. Human antibody production in transgenic mice: expression from 100 kb of the human IgH locus. *Eur. J. Immunol.* **21**, 1323–1328 (1991).
- Mendez, J. M. et al. Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat. Genet.* **15**, 148–158 (1997).
- Lanberg, N. et al. Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* **368**, 856–859 (1994).
- Nicholson, I. C. et al. Antibody repertoires of four- and five-allele translocus mice carrying human immunoglobulin heavy chain and kappa and lambda light chain yeast artificial chromosomes. *J. Immunol.* **163**, 6888–6905 (1999).
- Popov, A. V. et al. A human immunoglobulin lambda locus is similarly well expressed in mice and humans. *J. Exp. Med.* **188**, 1811–1820 (1999).
- Klingbeil, C. & Hsu, D. H. Pharmacology and safety assessment of humanized monoclonal antibodies for therapeutic use. *Toxicol. Pathol.* **27**, 1–3 (1999).

- Ishide, I. et al. Production of human monoclonal and polyclonal antibodies in transgenic animals. *Cloning Stem Cells* **4**, 91–102 (2002).
- McCreath, K. J. et al. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* **408**, 1088–1089 (2000).
- Danning, C. et al. Deletion of the α -(1,3)-galactosyl transferase (GCTA) gene and the prion protein (PrP) gene in sheep. *Nat. Biotechnol.* **18**, 550–562 (2001).
- Lai, L. et al. Production of α -(1,3)-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* **295**, 1089–1092 (2002).
- Ylan, D. et al. Targeted disruption of the α -(1,3)-galactosyltransferase gene in cloned pigs. *Nat. Biotechnol.* **20**, 251–255 (2002).
- Wall, R. J. New gene transfer methods. *Theriogenology* **57**, 189–201 (2002).
- Hamington, J. J. et al. Formation of de novo centromeres and construction of first-generation of human artificial microchromosomes. *Nat. Genet.* **15**, 345–355 (1997).
- Ikeno, M. et al. Construction of YAC-based mammalian artificial chromosomes. *Nat. Biotechnol.* **15**, 431–439 (1998).
- Shen, M. H. et al. Human mini-chromosomes in mouse embryonic stem cells. *Hum. Mol. Genet.* **6**, 1375–1382 (1997).

RESEARCH ARTICLE

17. Kuroiwa, Y. *et al.* Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts. *Nat. Biotechnol.* **18**, 1088–1090 (2000).
18. Loupart, M.L., Shen, M.H. & Smith, A. Differential stability of a human minichromosome in mouse cell lines. *Chromosome* **107**, 268–269 (1998).
19. Butler, J.E. Immunological diversity, B-cell and antibody repertoire development in large farm animals. *Rev. Sci. Tech.* **17**, 43–70 (1998).
20. Lucier, M.R. *et al.* Multiple sites of V_H diversification in cattle. *J. Immunol.* **161**, 5438–5444 (1998).
21. Meyer, A. *et al.* Immunoglobulin gene diversification in cattle. *Intern. Rev. Immunol.* **15**, 165–183 (1997).
22. Allken, R. *et al.* Structure and diversification of the bovine immunoglobulin repertoire. *Veterinary Immunol. Immunopathol.* **72**, 21–29 (1999).
23. Kuroiwa, Y. *et al.* *Gene Ther.*, in press.
24. Yamada, M. *et al.* Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood B lymphocytes. *J. Exp. Med.* **173**, 395–407 (1991).
25. Tizard, I.R. *Veterinary Immunology*, Edn. 5 (W.B. Saunders, Philadelphia, 1996).
26. Cibelli, J.B. *et al.* Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. *Nat. Biotechnol.* **16**, 642–646 (1998).
27. Cibelli, J.B. *et al.* Cloned transgenic calves produced from non-quiescent fetal fibroblasts. *Science* **280**, 1256–1258 (1998).
28. Kasanen, P. *et al.* Effect of fibroblast donor cell age and cell cycle on development of bovine nuclear transfer embryos *in vitro*. *Biol. Reprod.* **64**, 1467–1483 (2001).
29. Zakharenko, V. *et al.* Nuclear transfer in cattle with non-transfected and transfected fetal or cloned transgenic fetal and postnatal fibroblasts. *Mol. Reprod. Devel.* **60**, 362–369 (2001).
30. Tomizuka, K. *et al.* Functional expression and germline transmission of a human chromosome fragment in chimeric mice. *Nat. Genet.* **16**, 133–143 (1997).
31. Barsh, M.J., Knutsen, T. & Spurbach, J.L. *The AGY Cytogenetics Laboratory Manual* (Lippincott, Williams & Wilkins, New York, 1997).